

Chemical Carcinogen (3-Methylcholanthrene)-induced Pleomorphic Rhabdomyosarcomas in Fanconi Anemia *Fancd2*^{-/-}, *Fancg*^{-/-} (C57BL/6), *Fancd2*^{-/-} (129/Sv) Mice

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Abstract. *Background/Aim:* Radiation oncologists are reluctant to treat cancer in Fanconi Anemia (FA) patients due to their lack of homologous recombination repair of DNA strand breaks in normal tissues. To determine the therapeutic effects of irradiation and combination chemotherapy on cancer in syngeneic, radiosensitive FA mice, we derived transplantable cancers of the same genotype in three FA mouse strains. *Materials and Methods:* *Fancd2*^{-/-} mice on a C57BL/6 or Sv/129 background and *Fancg*^{-/-} mice (C57BL/6 background) that received 3-methylcholanthrene (3-MCA), were monitored for the development of subcutaneous tumors. *Results:* Tumors were induced at the site of 3-MCA injection, and tumor cell lines were established and found to be transplantable. Explanted tumors were identified as pleomorphic/rhabdomyosarcomas using immunohistochemical biomarkers. *Conclusion:* These transplantable FA mouse tumor cell lines should be valuable for testing effects of new radiation therapy protocols including FLASH high dose rate radiation delivery, immunotherapies, and combined radiation and chemotherapy treatments for radiosensitive FA patients.

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Fanconi Anemia (FA) represents a category of diseases in which subpopulations of patients are radiosensitive (1-6). FA patients are at risk of developing not only leukemia, but also solid tumors. FA patients are increasingly presenting with cancers in the head and neck region, esophagus, liver, and other tumor sites (7-11). Animal models of FA have been useful for evaluating new radiation protective drugs that might decrease the normal tissue toxicity of radiotherapy that is designed to treat cancer in FA patients (7-11). The increased incidence of squamous cell carcinomas in the aerodigestive tract of FA patients (12, 13), including both those who have successfully received bone marrow transplantation and those who have not, presents a challenge for the safe delivery of radiotherapy. Because some FA patients are radiosensitive due to defective DNA repair by homologous recombination, the delivery of standard of care chemoradiotherapy is not always possible for these patients (12, 13).

We and others have published that cancers in FA mice can be safely treated with fractionated irradiation (12, 13); however, the rapid onset and increased severity of normal tissue reactions including mucositis in radiotherapy, particularly in head and neck cancer treatment can be serious (12). The development of normal tissue radioprotectors using the current animal model systems of FA has been complicated by the need to transplant orthotopic tumors derived from wild type mice. We have tested the delivery of a radiation mitigator drug in models of FA mice, but the transplanted tumors were from wild type non-FA mice (9-11). Comparing tumor outcomes based on genotype within the same syngeneic mouse background would provide a more accurate system.

We previously published the first FA mouse tumor that was induced by the chemical carcinogen dimethylbenz[a] anthracene (DMBA) in *Fanca*^{-/-} (129/Sv) mice (14). That malignant tumor demonstrated properties of a rhabdomyosarcoma and was of *Fanca*^{-/-} mouse genotype (14).

To facilitate work with other FA mouse genotype models and provide data with cancers induced by a different chemical carcinogen, we used the chemical carcinogen 3-methylcholanthrene (3-MCA) (15, 16). In contrast to our prior studies with DMBA, which required multiple carcinogen applications, and subsequent administration of a tumor promoter (14), we found that administration of one injection of 3-MCA induced tumors at the injection site with no further need for multiple carcinogen or promoter injections.

We now report the generation of malignant tumors with three additional FA mouse strain models and in two different genetically distinct background mouse strains. The genetic abnormality in *Fancd2*^{-/-} mice is distinct from that in *Fanca*^{-/-} mice (2). We also used *Fancg*^{-/-} mice, which have a genetic abnormality that is distinct from either *Fanca*^{-/-} or *Fancd2*^{-/-} genotypes. Using *Fancd2*^{-/-} mice, we generated tumors in both 129/Sv mice (9) and the radiation resistant C57BL/6J mouse strain (10). *Fancg*^{-/-} (C57BL/6J) mice and *Fancd2*^{-/-} mice, the latter genotype in both the C57BL/6J and 129/Sv mouse genetic backgrounds, displayed tumor formation at the injection site of the chemical carcinogen 3-MCA. Tumor cell lines were derived by explanting excised tumors. Permanent transplantable cell lines were derived *in vitro*, and upon injection *in vivo*, these cell lines formed similar malignant tumors exhibiting histopathological and immunochemical features consistent with pleomorphic rhabdomyosarcomas. These three new transplantable tumor cell lines should be valuable for testing new chemoradiotherapy protocols in FA mice, considering the consistency of genotype between the cancer and normal tissues.

Such experiments should now provide a more realistic tissue environment for evaluating treatment outcomes including calculating the therapeutic ratio (tumor killing/normal tissue toxicity) of radiotherapy.

Materials and Methods

Experimental design. The goal of this project was to develop tumor cell lines from different strains of FA knockout mice. *Fancd2*^{-/-} knockout mice on the C57BL/6 background and *Fancg*^{-/-} knockout mice on the C57BL/6 background were obtained from Alan D'Andrea, MD, Dana Farber Cancer Institute, Boston, MA, USA. *Fanca*^{-/-} knockout mice on the 129/Sv background and *Fancd2*^{-/-} knockout mice on the 129/Sv background were obtained from Marcus Grompe, M.D., Oregon Health Science University, Portland, OR, USA. Female mice of each strain were injected subcutaneously with 200 µg 3-methylcholanthrene (3-MCA, #213942, Millipore/Sigma, Burlington, MA, USA) and monitored for tumor formation. The tumors were removed at 20 weeks, and half of each tumor was fixed in 10% paraformaldehyde (#047317, 9L, Thermo Fisher Scientific, Waltham, MA, USA). The remaining half of each tumor was prepared into a single cell suspension and tumor cell lines were established *in vitro*. Cells grown *in vitro* were injected at a concentration of 1×10⁷ cells in 100 µl saline into the dorsum of the

back of each respective mouse strain either C57BL/6NTac (Taconic Biosciences, Germantown, NY, USA), *Fand2*^{-/-} and *Fancg*^{-/-} mice, or 129/Sv mice for *Fancd2*^{-/-} (129/Sv) tumors. The fixed tumors were sectioned, and immunohistochemistry performed to identify the tumor phenotype.

Mice and animal housing. *Fancg*^{-/-} mice on the C57BL/6 background, *Fancd2*^{-/-} mice on the C57BL/6 background (8, 10), and *Fancd2*^{-/-} mice on the 129/Sv background (9) have been reported previously (11). *Fancg*^{-/-} mice on a C57BL/6 background have been reported (11).

C57BL/6NTac wild type mice were obtained from Taconic Biosciences (Germantown, NY, USA). All animals were female, housed four per cage, and fed standard laboratory chow, with access to deionized water. All experimental protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (PHS Assurance Number D16-00118). Veterinary care was provided by the University of Pittsburgh Division of Laboratory Animal Resources.

Chemical carcinogen 3-MCA administration and development of tumors at the 3-MCA injection site. Female *Fancd2*^{-/-} mice on either the C57BL/6 or the 129/Sv background, and *Fancg*^{-/-} mice on the C57BL/6 background were anesthetized using 2% isoflurane (#029405, Covetrus, Portland, ME, USA). The backs of the mice were shaved, epilated by application of Nair to the skin for 5 min, and the excess washed off using a water saturated paper towel. The mice were placed in their cages and allowed to recover by allowing the mice to breathe normal air. Twenty-four hours later, 3-MCA (Millipore Sigma, Cleveland, OH, USA) was dissolved in olive oil (#1478254, Millipore Sigma, Burlington, MA, USA) at 2 mg/ml, and 100 µl of the 3-MCA solution (prepared freshly the day of injection) was injected subcutaneously (200 µg per mouse) using a 1 ml syringe with a 28-gauge needle. Approximately 20 weeks after injection, tumors were identified at the site of injection and then monitored until they reached a diameter of 1 cm.

The tumors were excised, prepared into a single cell suspension by mincing the tumors using sterile surgical scissors and forceps. The cells were filtered through a series of smaller gauge needles beginning at 18 gauge and ending at 30 gauge. The cells were then filtered through a 0.45 µm syringe filter (FB14955206, Thermo Fisher Scientific) to remove cell clumps. The tumor cells were then plated in T-25 tissue culture flasks (#163371, Thermo Fisher Scientific) and grown in DMEM media (Dulbecco's Modified Eagles medium, #11965092, Thermo Fisher Scientific) containing 20% fetal calf serum (#900-208-500, GeminiBio, West Sacramento, CA, USA) and penicillin/streptomycin (5,000 U and 5 mg/ml) (P4458, Millipore Sigma, Burlington, MA, USA). Once cells on the flask surface reached confluence, they were passaged at ratios of 1:2, 1:5, and finally at 1:10. Aliquots of cells from each tumor cell line were frozen down at a concentration of 1×10⁶ cells/ml in Synth-a-Freeze cryopreservation medium (A1254201, Thermo Fisher Scientific) and stored in liquid nitrogen.

To demonstrate that the explanted isolated tumor cells were tumorigenic, aliquots of 1×10⁷ cells were injected subcutaneously into each of the five mice of each genotype corresponding to the tumor genotype. Male and female mice were used. The mice were followed for the development of tumors at the site of injection. Tumors were detected at injection sites by 2-3 weeks. When tumors

Table I. *Antibodies used in phenotyping tumors.*

Antibody	Supplier	Retrieval System	Detection System
SMA	Clone ASM-1 1:100 dilution, Millipore Sigma (Burlington, MA, USA)	Citrate Retrieval Solution, pH 6.0	Mouse on Mouse HRP Biocare Medical (Concord, CA, USA)
CD34	ARCO219-1:100 dilution, Invitrogen (Waltham, MA, USA)	Citrate Retrieval Solution, pH 6.0	Boost Rabbit, Cell Signaling Technology, (Danvers, MA, USA)
CD31	Clone: D8V9E-1:200 dilution, Cell Signaling Technology (Danvers, MA, USA)	Citrate Retrieval Solution, pH 6.0	Boost Rabbit, Cell Signaling Technology (Danvers, MA, USA)
MAC2	Clone: 1C1B2-1:1,000 dilution Protein Technology (Rosemont, IL, USA)	EDTA Retrieval Solution, pH 9.0	Mouse on Mouse HRP- Biocare Medical (Concord, CA, USA)
Desmin	Clone: Rabbit poly clonal cat# ab2272240-1:500 dilution Abcam (Boston, MA, USA)	Citrate Retrieval Solution, pH 6.0	Boost Rabbit (Cell Signaling Technology, Danvers, MA, USA)
Melan-a	Clone: EPR20380-1:500 dilution Abcam (Boston, MA, USA)	EDTA Retrieval Solution, pH 9.0	Boost Rabbit (Cell Signaling Technology, Danvers, MA, USA)
AE1/AE3	Clone: AE1/AE3=5D3-1:200 dilution Biocare Medical (Concord, CA, USA)	Citrate Retrieval Solution, pH 6.0	Mouse on Mouse HRP Biocare Medical (Concord, CA, USA)

reached 2 cm diameter, mice were sacrificed, and the tumors explanted. Each excised tumor was cut in half, with one half prepared into single cell suspensions and grown in tissue culture as described for the primary tumors. The other half of each tumor was fixed in 10% paraformaldehyde (#50-00-0, Thermo Fisher Scientific), paraffin embedded, sectioned, and stained with each of several antibodies to identify the phenotype of each tumor. The sections were stained with anti-SMA (smooth muscle cells), anti-CD34 (hematopoietic stem cells), anti-CD31 (endothelial cells), anti-MAC2 (histiocytic marker), anti-A1/A3 (epithelial cell marker for pan cytokeratin), anti-desmin (muscle), and anti-Melan-a (melanoma/melanocyte) (Table I).

Antigen retrieval was performed on each tissue section using a citrate retrieval solution (pH 6.0) or EDTA retrieval solution (pH 9.0, ab93678 or ab93680, Abcam, Waltham, MA, USA) in a Decloaking chamber NXGEN (Biocare Medical, Pacheco, CA, USA) at 120°C. The sections were stained in an Autostainer Plus (Dako, Carpinteria, CA, USA) using TBST rinse buffer (DAKO). A Horse Radish Peroxidase (HRP)-labelled polymer was used for detection. 3,3', Diaminobenzidine+ substrate (DAKO) was used as the substrate. Hematoxylin (DAKO) was used as a counterstain.

The antibodies used for the tumor phenotyping are listed in Table I. To phenotype the *Fancd2*^{-/-} (129SV background) and *Fancd2*^{-/-} (C57BL/6 background), or *Fancg*^{-/-} tumors in the C57BL/6 background, tumor sections from explanted tumors were prepared. These sections were stained with the antibodies listed in Table I and evaluated by a board-certified veterinarian pathologist (L.H.R.).

Immunohistochemical staining. The methods for immunohistochemical staining using antibodies for each biomarker for carcinoma and sarcoma were as previously published (14). We analyzed the histopathology of the primary tumors and each of the successive transplant generation tumors. A 2 cm tumor was excised

from the tumor bearing mice, fixed in 10% paraformaldehyde, and paraffin embedded. The tumor was sectioned and stained using hematoxylin and eosin, as well as for markers to identify the phenotype. To further characterize tumors formed by *Fanca*^{-/-} tumor cell lines, and clonal sublines, tumor cell sections were immunocytochemically stained with the antibodies listed in Table I including: SMA (smooth muscle), S100 (Schwann cell/melanocyte), CD34 (hematopoietic stem cell), MAC2 (histiocytic marker), CD31 (endothelial), Melan-a (melanoma), Desmin (skeletal/cardiac muscle), and A1/A3 (epithelial cells). Details on the antibodies, such as supplier, dilution, and antigen retrieval are also shown in Table I. The slides were deparaffinized and rehydrated using a standard histology protocol. Antigen retrieval was performed using a citrate retrieval solution, pH 6.0, or EDTA retrieval solution, pH 9.0 (ab93678 or ab93680, Abcam) and a Decloaking chamber NXGEN (Biocare Medical) at 120°C. The slides were stained using an Autostainer Plus (Dako) with TBST rinse buffer (Dako). Each antibody was applied as described in Table I. The detection system was an HRP-labeled polymer, as listed in Table I. The substrate used was 3,3', Diamino-benzidine+(Dako). The slides were then counterstained with Hematoxylin (Dako).

Cloning single-cell-derived cell lines from explanted tumors. Subclonal lines of each tumor cell line were established by preparing a single cell suspension of each tumor cell line. A flow cytometer was used to plate one cell into each well of five 96 well tissue culture plates (#260887, Thermo Fisher Scientific). Each well contained 100 µl of DMEM media containing 10% FBS and Penicillin and Streptomycin as described above. The cells in each well were observed daily and once the cells became confluent in that particular well, they were transferred each to one well of a 24 well plate (#142475, Thermo Fisher Scientific). Once the cells reached confluency, each well was then prepared into a single cell

suspension and transferred to a T25 tissue culture flask (#163371, Thermo Fisher Scientific). The cells were then expanded and frozen down at 1×10^6 cells per ml in a 1 ml cryopreservation tube (#347783, Thermo Fisher Scientific) in Synth-a-Freeze cryopreservation medium (A1254201, Thermo Fisher Scientific) and frozen in -80°C freezer. After freezing, the cells were transferred into a liquid nitrogen dewar for storage.

Results

Appearance of tumors at each 3-MCA injection site in each FA mouse strain. We previously published that delivery of the carcinogen 4-nitroquinolone oxide (4-NQO) in drinking water and prolonged exposure for twenty weeks induced tumor formation in K14E7Fancd2^{-/-} mice (17) (Figure 1). We also reported that subcutaneous injections of the carcinogen N, N-Dimethylbenzylamine (DMBA, #185582, Millipore Sigma) followed by weekly subcutaneous administration of the tumor promoter 12-O-tetradecanoylphorbol 13-acetate (TPA, P1585, Millipore Sigma) was required for tumor induction in Fancd2^{-/-} (129/Sv) mice (14). Prior publication showed that a single injection of the carcinogen 3-MCA effectively induced tumors at the injection site (15, 16). To facilitate more rapid generation of tumors, we tested the effect of injections of 3-MCA in each of the three FA mouse strains: Fancd2^{-/-} on the C57BL/6 background, Fancd2^{-/-} on the 129/Sv background, and Fancg^{-/-} mice on the C57BL/6 background. The appearance of tumors at the injected site of 3-MCA is shown for each of the three FA genotypes in Figure 2. Transplantation of excised tumors to a second-generation of each syngeneic mouse strain was next performed (Figure 3). Excised tumors that formed at the injection sites with intact skin are shown in Figure 2 and Figure 3.

Excised tumors from each mouse strain were next prepared into single cell suspensions and grown *in vitro* in DMEM tissue culture medium. Figure 4 demonstrates the appearance of cultures of tumor cells for each of the three tumor genotypes after explanting tumors from 3-MCA treated Fancd2^{-/-} (C57BL/6), Fancd2^{-/-} (129/Sv) mouse, and Fancg^{-/-} (C57BL/6).

Initially, tumor cells grew as flat monolayers (Figure 4). Over time, round clumps of tumor cells that piled up on these flat monolayers were formed by cells from each tumor genotype (Figure 5).

Histopathologic and immunohistochemical diagnosis of explanted tumor. Hematoxylin and Eosin (H & E) staining of tumor cells and immunohistochemical staining results are summarized in Table II. In summary, tumors were densely cellular and composed of tightly packed and moderately to highly pleomorphic spindle cells arranged in disorganized interlacing bundles and streams within a scant collagenous matrix. The mitotic rate averaged 5-7 per 40×field. Rarely, these pleomorphic tumor cells were seen in areas with

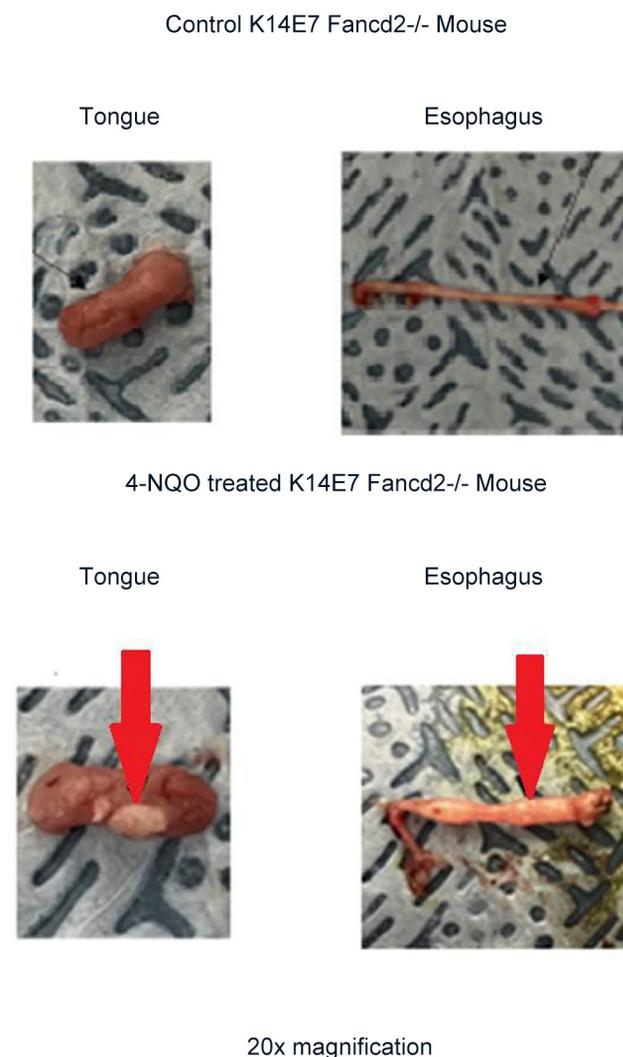


Figure 1. *In vivo* appearance of K14E7Fancd2^{-/-} tumors induced by oral 4-NQO. K14E7Fancd2 mice were administered drinking water containing 4-NQO for 20 weeks (17). By week 35 to week 40, tumors in the esophagus and oral cavity were detected (arrow).

mature skeletal muscle and features indicating differential skeletal muscle in otherwise highly pleomorphic regions. This is illustrated in Figure 6. Table I and Table II define the characteristics of the immunostaining of the tumors.

Immunostaining was consistent with the diagnosis of pleomorphic rhabdomyosarcoma with explanted tumors from each of the three genotypes. Briefly, tumor cells from all three cell lines revealed strong immunostaining for desmin, variably strong staining for SMA and CD34, and no immunostaining for S100, CD31, MelanA, MAC2, or pan cytokeratin AE1/AE3. These features, combined with the histologic features, allow for the diagnosis of pleomorphic rhabdomyosarcoma.

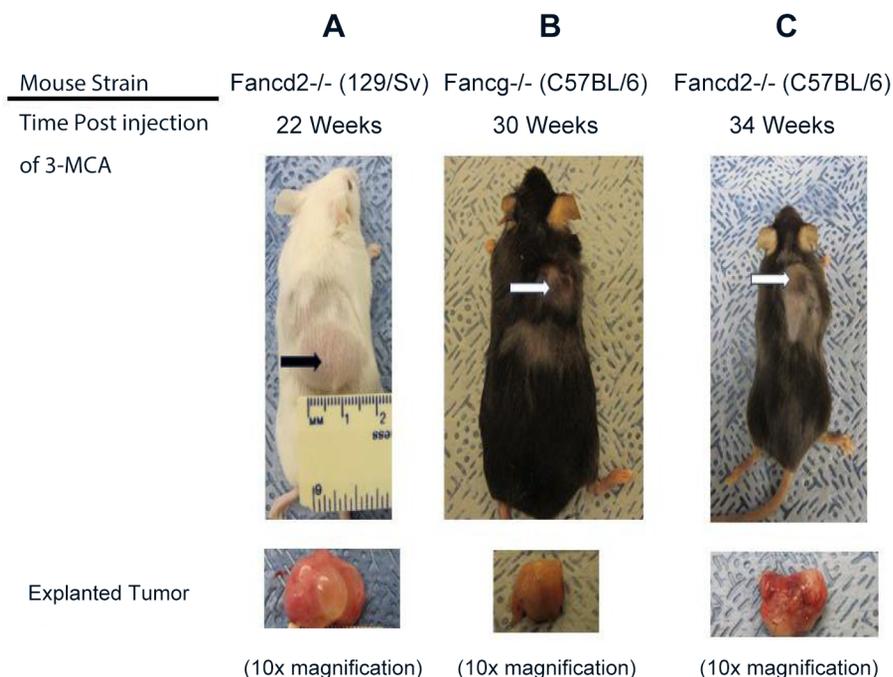


Figure 2. *In vivo* appearance of tumors induced in Fancd2^{-/-} (129/Sv), Fancg^{-/-} (C57BL/6) and Fancd2^{-/-} (C57BL/6) mice by 3-MCA injection. The dorsum of each Fancd2^{-/-} (129/Sv) (A), Fancg^{-/-} (C57BL/6) (B) and Fancd2^{-/-} (C57BL/6) (C) mouse was epilated by shaving and treatment with Nair. Twenty-four hours later, each mouse was injected subcutaneously with 3-MCA as described in the Methods. Twenty to twenty-five weeks later, tumors formed at the injection site (arrow). The tumors were then explanted and divided into two halves for either culture *in vitro* for the development of cell lines (Figure 4 and Figure 5) or prepared for immunohistochemistry (Table I and Table II).

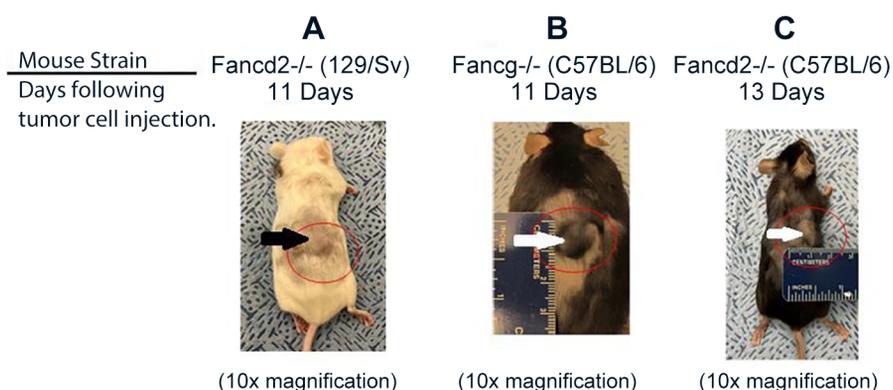


Figure 3. *In vivo* appearance of second passage of tumors transplanted into syngeneic mice from the cells that were explanted from primary tumors shown in Figure 2. Tumors were explanted and prepared into single cell suspensions to develop cell lines. To demonstrate that the second-generation tumor cell lines were transplantable, each cell line was injected (1×10^7 cells in 100 μ l of PBS) subcutaneously into the dorsum of epilated mice of the same genotype. Two to three weeks later the tumors were detected at the area of injection (arrow).

Tumor cell line cloning. Tumor cells from each genotype were subjected to flow cytometry to establish more stable clonal cell lines. Each subclone was injected into mice of the same genotype to examine whether the cloned cell lines retained their ability to grow *in vivo*. Thus, we demonstrated that the injected clonal cell lines grew in mice of the same genotype.

Discussion

The present report extends our published results on the generation of tumors by chemical carcinogen injection in Fancd2^{-/-} (129/Sv) mice to include three additional FA mouse strains. The tumors in Fancg^{-/-} (C57BL/6), Fancd2^{-/-}

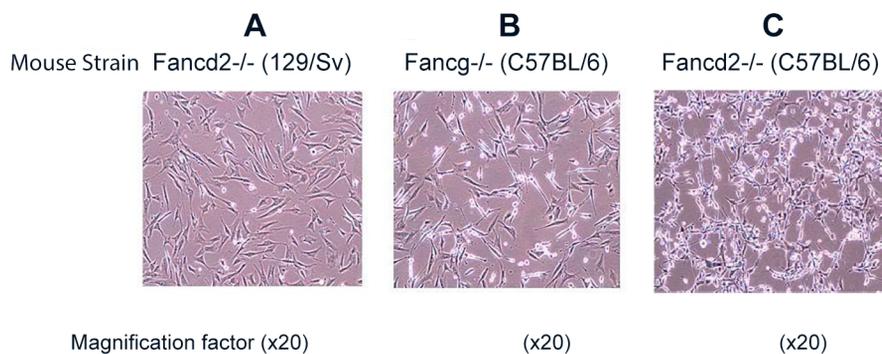


Figure 4. *In vitro* appearance of explanted tumors from 3-MCA injected mice. Tumors were explanted from mice injected with 3-MCA (Figure 2), prepared into single cell suspensions, and then plated in DMEM media in T24 tissue culture flasks. Figure 4A-C shows the *in vitro* appearance of each established cell line (magnification factor $\times 20$).

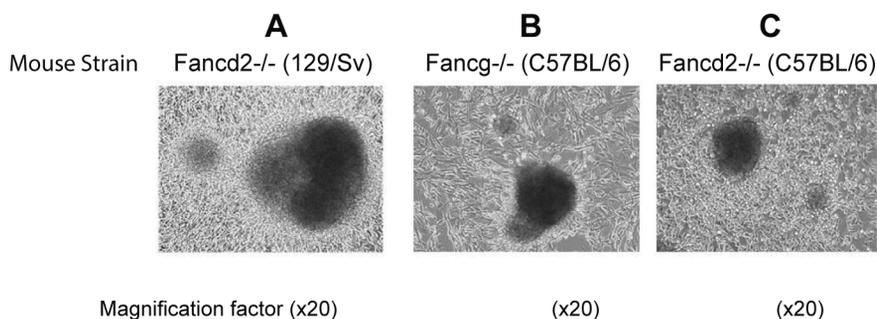


Figure 5. *In vitro* appearance of tumor nodules evolving *in vitro* from cultures of passaged tumor cell lines. Tumor cell lines explanted from 3-MCA induced tumors on *Fancd2*^{-/-} (129/Sv) (A), *Fancg*^{-/-} (C57BL/6) (B) and *Fancd2*^{-/-} (C57BL/6) (C) were photographed when approaching confluence in culture. Each tumor cell line formed nodules of malignant cells (magnification factor $\times 20$).

(C57BL/6), and *Fancd2*^{-/-} (129/Sv) mice all revealed features of pleomorphic rhabdomyosarcomas following injection of the chemical carcinogen 3-MCA (15, 16). The present results confirm and extend prior publication showing chemical carcinogen-induced sarcoma tumors in *Fanca*^{-/-} (129/Sv) mice using the chemical carcinogen DMBA (14). DMBA-induced tumors in *Fanca*^{-/-} mice also had histopathologic characteristics of pleomorphic rhabdomyosarcomas.

The present tumor cell lines derived from three different FA mouse strains. Each mouse was injected with 3-MCA, and each demonstrated a soft tissue sarcoma phenotype. The characteristics of growth of the tumor cell lines from each of these three mouse strains were similar to those in a previous publication (14). Tumor cells grew in a flat monolayer and round clumps of tumor cells grew from the monolayers and either remained attached or floated into the medium. Cloning of each of the cell lines revealed subclones of single cell origin that had the same characteristics of the parent tumor. Immunohistochemistry demonstrated biomarkers of pleomorphic rhabdomyosarcoma, a subtype of soft tissue sarcoma.

These three new tumor cell lines provide a valuable resource by which to transplant tumors into mice with the same FA genotype as that of the tumors. This system facilitates the study of the effects of radiotherapy, chemotherapy or combination therapies including immunotherapy, on the biologic response of tumors in the setting of the same genotype as the surrounding normal tissues. Intravenous injection of tumor cell lines should provide a model for metastasis in various organs and the ability to use small animal irradiators delivering stereotactic radiosurgery in the setting of the FA tissue of the same genotype. These transplantable sarcoma cell lines will allow study of the best fractionation schemes and modalities of radiotherapy including proton therapy (18) and FLASH (19) radiotherapy to minimize normal tissue damage in FA mouse models and eventually translate the findings for use in FA patients. Previous studies have demonstrated that a therapeutic trial of low-dose, small-volume radiotherapy can differentiate between FA patients who are radiosensitive and those who are not (12, 13). However, patients who are radiosensitive may benefit from personalized treatments that have yet to be designed.

Table II. Results of immunohistochemistry analysis of tumor tissues from 3-MCA-induced tumors in *Fancd2*^{-/-} (129/Sv), *Fancg*^{-/-} (C57BL/6), and *Fancd2*^{-/-} (C57BL/6) mice.

	SMA	CD34	S100	CD31	Melan A	Desmin	MAC2	AE1/AE3
<i>Fancd2</i> ^{-/-} 129SV-1	-	+	-	-	-	+++	-	-
<i>Fancg</i> ^{-/-} -C57-1	+	+	-	-	-	+++	-	-
<i>Fancg</i> ^{-/-} -C57-2	++	+	-	-	-	+++	-	-
<i>Fancd2</i> ^{-/-} -C57- 1	++	++	-	-	-	+++	-	-
<i>Fancd2</i> ^{-/-} -C57- 2	++	++	-	-	-	+++	-	-
<i>Fancd2</i> ^{-/-} -C57- 3	++	++	-	-	-	+++	-	-
<i>Fancd2</i> ^{-/-} -C57-1-12	++	++	-	-	-	+	-	-

All tumors show moderate to strong expression of desmin within neoplastic cells, variably strong expression of SMA and CD34, and no expression of S100, CD31, MelanA, MAC2, or AE1/AE3. These data combined with histologic features (Figure 6) are consistent with the diagnosis of pleomorphic rhabdomyosarcoma.

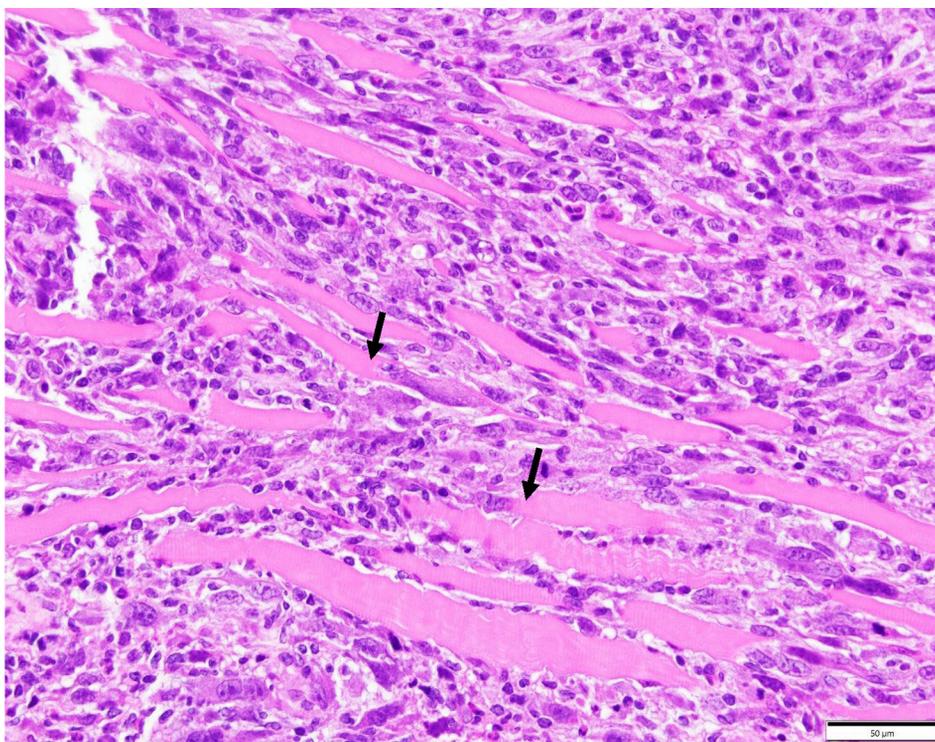


Figure 6. Histological appearance of the transition zone from skeletal to sarcoma in an explanted tumor from a 3-MCA-injected mouse in Figure 2B. (Animal *Fancg*^{-/-}1, Magnification factor $\times 40$, Scale bar=50 μm). A tumor from a *Fancg* (C57BL/6) mouse demonstrated pleomorphic spindle cells with rare mature skeletal muscle cells containing large pleomorphic nuclei. (Magnification factor $\times 40$, Scale bar=50 μm)

In previous studies with FA mouse models in *Fanca*^{-/-}, *Fancg*^{-/-} mice, and *Fancd2*^{-/-} mice, it has been demonstrated that oral delivery of a radioprotector drug (JP4-039) minimized normal tissue mucositis. These studies were performed in FA mice in which orthotopic tumors (derived from wild type mice) were implanted into the oral cavity of FA mice (9-11). These studies demonstrated that fractionated radiotherapy can be safely given to FA mice if they are treated

with intraoral administration of a radioprotector/radiation mitigator drug, JP4-039, which is a mitochondrial targeted GS-nitroxide (20) and provides targeted mitochondrial protection from radiation-induced apoptosis. These prior studies were all carried out with orthotopic tumors that were derived from a head and neck cancer cell line that had been developed from wild type mice (9-11). The published results were interesting and provocative and provide information for

the safe administration of a radioprotector drug to FA mice and potentially to FA patients in the future. However, the studies were imperfect because the genotype of the tumor was not the same as that of the background FA mouse strain.

Cancers that were derived in FA mice have been reported previously (3-5), but these publications did not include the derivation of transplantable permanent cell lines. We first reported the development of a permanent transplantable FA mouse sarcoma cell line (14) in *Fanca*^{-/-} mice.

We also reported the development of a malignant plasmacytoma permanent cell line derived from *K14E7Fancd2*^{-/-} mice by oral delivery of 4-NQO (17). The present report now makes available three additional tumors that are derived from pure FA knockout mice. Furthermore, our two prior publications (14, 17) plus the present report, together represent induction of cancer in FA mice by three different chemical carcinogens 4-NQO, DMBA, and 3-MCA.

The treatment of FA mice with these newly established transplantable cancer cell lines will allow testing of new therapeutics, which will be particularly intriguing to evaluate (18, 19) now with syngeneic tumors. The FA genotype is known to show greater senescence of cells in irradiated tissues (21). Furthermore, the role of FA associated oncogenes and TGF- β in mice may alter the formation of tumor metastasis, recurrence, and alter radiosensitivity (22-24). New experiments and data can now be generated using tumors with the same genotype as the surrounding normal tissues. The present report provides the availability of three new FA tumors derived from each of three different FA gene models in addition to our prior publications of a DMBA carcinogen- induced tumor in *Fanca*^{-/-} mice (14). We now provide resources for carrying out studies to test the effect of new treatment modalities in tumors derived from FA mice in the same genotype background as the normal tissues. These three new FA tumor cell lines derived from FA mouse genotypes of two genes (*Fancd2* and *Fancg*) in the FA pathway are also now available for researchers interested in developing protocols to treat tumors in FA patients while reducing toxicity.

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Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

J.G. designed the studies, M.E. prepared 3-MCA for injection, R.F. carried out animal studies, D.S. performed the cell line characterization, A.G. made tissue sections and stained sections for

immunohistochemistry, L.H.R. carried out the histology and immunohistochemistry analysis and A.M. edited and reviewed the manuscript.

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